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Attorney Docket No.: 5835.200-US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Bisgård-Frantzen et al Serial No.: 09/710,339

Confirmation No: 9183 Group Art Unit: To be assigned

Filed: November 9, 2000 Examiner: To be assigned

For: Fungamyl-like Alpha-Amylase Variants

CERTIFICATE OF MAILING UNDER 37 CFR 1.8(a)

Commissioner for Patents
Washington, DC 20231

Sir:

I hereby certify that the attached correspondence comprising:

1. Claim to Convention Priority

is being deposited with the United States Postal Service as first class mail in an envelope addressed to:

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CLAIM TO CONVENTION PRIORITY UNDER 35 U.S.C. 119

Commissioner for Patents
Washington, DC 20231

Sir:

In the matter of the above-identified application and under the provisions of 35 U.S.C. 119 and 37 C.F.R. 1.55, Applicant(s) claim priority of Danish application no. PA 1999 01617 filed on November 10, 1999. Applicant(s) submit(s) a duly certified copy of said foreign application.

Respectfully submitted,

Date: July 25, 2001

Elias J. Lambiris, Reg. No. 33,728
Novozymes North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10174-6401
(212) 867-0123

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JUL 30 2001
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Kongeriget Danmark

Patent application No.:	PA 1999 01617
Date of filing:	10 November 1999
Applicant:	Novo Nordisk A/S Novo Allé DK-2880 Bagsværd

This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following information:

- The specification, claims, abstract and sequence listing as filed with the application on the filing date indicated above.

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Erhvervsministeriet

TAASTRUP 27 April 2001

Karin Schlichting
Head Clerk

Modtaget PD
10 NOV. 1999

TITLE: Fungamyl-like Alpha-Amylase Variants

FIELD OF THE INVENTION

The present invention relates to novel α-amylase variants (mutants) of Fungamyl-like α-amylases, in particular with improved thermal stability at acidic pH. The invention also relates to the use of such variants.

BACKGROUND OF THE INVENTION

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

There is a very extensive body of patent and scientific literature relating to this industrially very important class of enzymes. A number of α-amylase referred to as "Termamyl-like α-amylases" and variants thereof are known from, *e.g.*, WO 90/11352, WO 95/10603, WO 95/26397, WO 96/23873 and WO 96/23874. Termamyl-like α-amylases are very thermostable and therefore suitable for processes carried out at high temperatures such as starch liquefaction in dextrose production processes.

Another group of α-amylases are referred to as "Fungamyl-like α-amylases", which are α-amylases related to the α-amylase derived from *Aspergillus oryzae* (and shown in SEQ ID NO: 1). These Fungamyl-like α-amylases have a relatively low thermostability (FUNGAMYL® has a optimum around 55°C) and is therefore not suitable for processes carried out at high temperatures. Fungamyl-like α-amylases are today used for making syrups for, e.g., the brewing industry. Such processes are operated at around 60°C resulting in that usually in the range of double the enzyme dosage must be used to compensate for the low thermostability. Further, at 55°C infection problems may occur. As such processes today furthermore are carried out at a pH of 5.5, instead of, e.g., pH 4.5, pH adjustment and addition of Sodium to the syrups are necessitated.

Therefore, it would be advantageous to provide a Fungamyl-like α-amylase with increased thermostability preferably at an acidic pH.

BRIEF DISCLOSURE OF THE INVENTION

The object of the present invention is to provide Fungamyl-like α-amylase variant, in particular with improved thermostablility especially at acidic pHs.

The term "an α-amylase variant with improved thermostability" means in the context of the present invention an α-amylase variant which has a higher thermostability than corresponding parent α-amylases. The determination of thermostability is described below in the Materials and Method section.

The inventors have provided a number of improved Fubgal-like α-amylase variants as will be described further below.

DETAILED DISCLOSURE OF THE INVENTION

A goal of the work underlying the present invention was to improve the thermal stability at acidic pH of Fungamyl-like α-amylases.

<u>Identifying positions and/or regions to be mutated to obtain improved thermostability</u>

Molecular dynamics (MD) simulations indicate the mobility of the amino acids in the protein structure (see McCammon, JA and Harvey, SC., (1987), "Dynamics of proteins and nucleic acids". Cambridge University Press.). Such protein dynamics are often compared to the crystallographic B-factors (see Stout, GH and Jensen, LH, (1989), "X-ray structure determination", Wiley). By running the MD simulation at different protonation states of the titrateable residues, the pH related mobility of residues are simulated. Regions having the highest mobility or flexibility (here isotropic fluctuations) are selected for random mutagenesis. It is here understood that the high mobility found in certain areas of the protein, can be thermally improved by substituting residues in these residues. The substitutions are directed against residues that have bigger side-chains and/or which have capability of forming improved contacts to residues in the near environment. The Fungamyl α-amylase shown in SEQ ID NO: 1 derived from *Aspergillus oryzae* was used for the MD simulation.

Regions found by Molecular dynamics (MD) simulations to be suitable for mutation when wanting to obtain improved thermal

stability are the following:
Region 98-110,
Region 150-160,
Region 160-167,
Region 280-288,
Region 448-455,
Region 468-475.

The above regions are show to be flexible. Making said regions more rigid would make the molecule more thermostable. Specific substitutions include one or more of the following:
Q153S

Accordingly, in a first aspect the present invention relates to a variant of a parent Fungamyl-like α-amylase comprising one or more mutations in the regions and positions described further below.

Nomenclature

In the present description and claims, the conventional one-letter and three-letter codes for amino acid residues are used. For ease of reference, α-amylase variants of the invention are described by use of the following nomenclature:
Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of alanine for asparagine in position 30 is shown as:

Ala30Asn or A30N

a deletion of alanine in the same position is shown as:

Ala30* or A30*

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala30AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)* or Δ(A30-N33).

Where a specific α-amylase contains a "deletion" in comparison with other α-amylases and an insertion is made in such a position this is indicated as:

*36Asp or *36D

for insertion of an aspartic acid in position 36
Multiple mutations are separated by plus signs, i.e.:

Ala30Asp + Glu34Ser or A30N+E34S

representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively. Multiple mutation may also be separated as follows, i.e., meaning the same as the plus sign:

Ala30Asp/Glu34Ser or A30N/E34S

When one or more alternative amino acid residues may be inserted in a given position it is indicated as

A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine in position 30 is mentioned, but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of:

R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

Fungamyl-like α-amylases

Parent Fungamyl-like α-amylase are according to the present invention enzymes with α-amylase activity which either have at least 60%, preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, even more preferably at least 93%, even more preferably at least 95%, even more preferably at least 97%, even more preferably at least 99% identity to SEQ ID NO: 1 and/or structurally resembles the three-dimensional structure of the Fungamyl® α-amylase shown in SEQ ID NO: 1.

Commercial parent Fungamyl-like α-amylases

Commercially available parent Fungamyl-like α-amylases include Fungamyl® from Novo Nordisk. Fungamyl is an fungal α-amylase obtained from a selected strain of *Aspergillus oryzae*. In the starch industry, Fungamyl® is used for production of high maltose syrups, 45-60% maltose (2-7% glucose) or high conversion syrups, DE 60-70, 35-43% glucose, 30-37% maltose.

In the brewing industry, Fungamyl® is added during fermentation in order to increase fermentability of the wort.

In the alcohol industry, Fungamyl® may be used for liquefaction of starch in a distillery mash if the existing equipment favours low-temperature liquefaction (55-60°C). Fungamyl® is also used for baking and can be used for all types of bread and baked products. For instance Fungamyl® improves the dough stability, result in greater bread volume, improves crumb softness and give the crust a darker color.

α-amylase variants of the invention

In the first aspect the invention relates to a variant of a parent Fungamyl-like α-amylase comprising one or more mutation(s) in the following positions(s) or region(s) in the amino acid sequence shown in NO: 1:

Region 98-110,

Region 150-160,

Region 280-288,

Region 448-455,

Region 468-475, and/or in a corresponding position or region in a homologous Fungamyl-like α-amylase which displays at least 60% identity with the amino acid sequences shown in SEQ ID NO: 1.

In an embodiment the region mutated is Region 98-110.

In an embodiment the region mutated is Region 150-160. Within this region the following mutation(s) is(are) specifically contemplated: Q153S

In an embodiment the region mutated is Region 280-288.

In an embodiment the region mutated is Region 468-475.

Homology (Identity)

The homology referred to above of the parent glucoamylase is determined as the degree of identity between two protein sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, p. 443-453). Using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP

extension penalty of 0.1, the mature part of a polypeptide encoded by an analogous DNA sequence of the invention exhibits a degree of identity preferably of at least 60%, such as 70%, at least 80%, at least 90%, more preferably at least 95%, more preferably at least 97%, and most preferably at least 99% with the mature part of the amino acid sequence shown in SEQ ID NO: 1.

In a preferred embodiment the variant of the invention has improved thermal stability at especially acidic pH.
The term "acidic pH" means in the a pH of 4-5, in particular 4.2-4.7.

Cloning a DNA sequence encoding an Fungamyl-like α-amylase

The DNA sequence encoding a parent Fungamyl-like α-amylase may be isolated from any cell or microorganism producing α-amylases, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α-amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, labeled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known α-amylase gene could be used as a probe to identify α-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying α-amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for α-amylase (*i.e.*, maltose), thereby allowing clones expressing the α-amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, *e.g.* the phosphoroamidite method described S.L. Beaucage and M.H. Caruthers, (1981), Tetrahedron Letters 22, p. 1859-1869, or the method described by Matthes et al., (1984), EMBO J. 3, p. 801-805. In the phosphoroamidite method, oligonucleotides are synthesized, *e.g.,* in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., (1988), Science 239, 1988, pp. 487-491.

Site-directed mutagenesis

Once an Fungamyl-like α-amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. In a specific method, a single-stranded gap of DNA, the α-amylase-encoding sequence, is created in a vector carrying the α-amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984), Biotechnology 2, p. 646-639. US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into α-amylase-encoding DNA sequences is described in Nelson and Long, (1989), Analytical Biochemistry 180, p. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random Mutagenesis

Random mutagenesis is suitably performed either as localized or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

The random mutagenesis of a DNA sequence encoding a parent glucoamylase may be conveniently performed by use of any method known in the art.

In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a parent Fungamyl-like α-amylase, wherein the variant exhibits increased thermal stability, especially at acidic pH, relative to the parent, the method comprising:

(a) subjecting a DNA sequence encoding the parent Fungamyl-like α-amylase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing an α-amylase variant which has an altered property (i.e. thermal stability) relative to the parent Fungamyl-like α-amylase.

Step (a) of the above method of the invention is preferably performed using doped primers, as described in the working examples herein (vide infra).

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, *e.g.*, be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the glucoamylase enzyme by any published technique, using *e.g.* PCR, LCR or any DNA polymerase and ligase as deemed appropriate.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and mutation in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, *e.g.*, so as to allow for the introduction of 90% wild type and 10% mutations in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, *inter alia*, ensures that introduction of stop codons is avoided.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent glucoamylase is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the glucoamylase by, *e.g.*, transforming a plasmid containing the parent glycosylase into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mu-ta-ted plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may be conveniently present in a genomic or cDNA library prepared from an organism expressing the parent glucoamylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or other-wise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus;* and gram-negative bacteria such as *E. coli.*

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis

The random mutagenesis may be advantageously localized to a part of the parent α-amylase in question. This may, *e.g.*, be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, *e.g.*, by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

Alternative methods for providing variants of the invention include gene shuffling *e.g.* as described in WO 95/22625 (from Affymax Technologies N.V.) or in WO 96/00343 (from Novo Nordisk A/S).

Expression of α-amylase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

Expression vector

The recombinant expression vector carrying the DNA sequence encoding an α-amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. The vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Examples of suitable expression vectors include pMT838.

Promoter

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α-amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, the TPI (triose phosphate isomerase) promoter from *S. cerevisiae*

(Alber et al. (1982), J. Mol. Appl. Genet 1, p. 419-434, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

Expression vector

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, *e.g.*, as described in WO 91/17243.

The procedures used to ligate the DNA construct of the invention encoding a glucoamylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

Host Cells

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a glucoamylase variant of the invention. The cell may be

transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, *e.g.* by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g., a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are Gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram-negative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, *e.g.*, *Saccharomyces cerevisiae*.

The host cell may also be a filamentous fungus *e.g.* a strain belonging to a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger, or* a strain of *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gribberella zeae,* previously *Sphaeria zeae, synonym with Gibberella roseum and Gibberella roseum* f. sp. *cerealis)*, or *Fusarium sulphureum (*in the prefect state named *Gibberella puricaris,* synonym with *Fusarium trichothecioides, Fusarium bactridioides, Fusarium sambucium, Fusarium roseum,* and *Fusarium roseum var. graminearum), Fusarium cerealis (*synonym with *Fusarium crokkwellnse), or Fusarium venenatum.*

In a preferred embodiment of the invention the host cell is a protease deficient or protease minus strain. This may for instance be the protease deficient strain of the genus *Aspergillus,* in particular a strain of *A. oryzae, such as A.*

oryzae JaL125 having the alkaline protease gene named "alp" deleted. This strain is described in WO 97/35956 (Novo Nordisk).

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host micro-organism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

Method of producing an α-amylase variant of the invention

In a yet further aspect, the present invention relates to a method of producing an α-amylase variant of the invention, which method comprises cultivating a host cell under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α-amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (*e.g.,* as described in catalogues of the American Type Culture Collection).

The α-amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Starch conversion

The present invention provides a method of using α-amylase variants of the invention for producing glucose or maltose or the like from starch.

Generally, the method includes the steps of partially hydrolyzing precursor starch in the presence of α-amylase and then further hydrolyzing the release of D-glucose from the non-reducing ends of the starch or related oligo- and polysaccharide molecules in the presence of glucoamylase by cleaving α-(1→4) and α-(1→6) glucosidic bonds.

The partial hydrolysis of the precursor starch utilizing α-amylase provides an initial breakdown of the starch molecules by hydrolyzing internal α-(1→4)-linkages. In commercial applications, the initial hydrolysis using α-amylase is run at a temperature of approximately 105°C. A very high starch concentration is processed, usually 30% to 40% solids. The initial hydrolysis is usually carried out for five minutes at this elevated temperature. The partially hydrolyzed starch can then be transferred to a second tank and incubated for approximately one hour at a temperature of 85° to 90°C to derive a dextrose equivalent (D.E.) of 10 to 15.

The step of further hydrolyzing the release of D-glucose from the non-reducing ends of the starch or related oligo- and polysaccharides molecules in the presence of glucoamylase is normally carried out in a separate tank at a reduced temperature between 30° and 60°C. Preferably the temperature of the substrate liquid is dropped to between 55° and 60°C. The pH of the solution is dropped from 6 to 6.5 to a range between 3 and 5.5. Preferably, the pH of the solution is 4 to 4.5. The glucoamylase is added to the solution and the reaction is carried out for 24-72 hours, preferably 36-48 hours.

By improving the thermo stability of the Fungamyl-like α-amylase variant according to the invention said α-amylases may be used for starch liquefaction.

In an aspect the invention relates to the use of a α-amylase variant of the invention in a starch conversion process.

The α-amylase variant of the invention may also be used in a process for producing alcohol such as ethanol for fuel or beverage.

The α-amylase variant of the invention may also be used in baking processes.

The α-amylase variant of the invention may also be used in brewing processes.

MATERIALS AND METHODS

Material:

Enzymes:

FUNGAMYL®: available from Novo Nordisk and Shown in SQ ID NO: 1.

Host cell:

A. oryzae JaL125: *Aspergillus oryzae* IFO 4177 available from Institute for Fermention, Osaka; 17-25 Juso Hammachi 2-Chome Yodogawa-ku, Osaka, Japan, having the alkaline protease gene named "alp" (described by Murakami K et al., (1991), Agric. Biol. Chem. 55, p. 2807-2811) deleted by a one step gene replacement method (described by G. May in "Applied Molecular Genetics of Filamentous Fungi" (1992), p. 1-25. Eds. J. R. Kinghorn and G. Turner; Blackie Academic and Professional), using the *A. oryzae* pyrG gene as marker. Strain JaL 125 is further disclosed in WO 97/35956 (Novo Nordisk).

Micro-organisms:

Strain: Saccharomyces cerevisiae YNG318: MATαleu2-Δ2 ura3-52 his4-539 pep4-Δ1[cir+]

Methods:

Determination of FAU activity

One Fungal Alpha-Amylase Unit (FAU) is defined as the amount of enzyme which breaks down 5.26 g starch (Merck Amylum solubile Erg. B.6, Batch 9947275) per hour at Novo Nordisk's standard method for determination of alpha-amylase based upon the following standard conditions:

Substrate. . . . . . soluble starch

Temperature. . . . . 37°C

pH. 4.7

Reaction time. . . . 7-20 minutes

A detailed description of Novo Nordisk's method is available on request.

<u>Transformation of *Aspergillus oryzae* (general procedure)</u>

100 ml of YPD (Sherman et al., (1981), Methods in Yeast Genetics, Cold Spring Harbor Laboratory) are inoculated with spores of *A. oryzae* and incubated with shaking for about 24 hours. The mycelium is harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium is suspended in 15 ml of 1.2 M $MgSO_4$, 10 mM NaH_2PO_4, pH 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym™ 234 is added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) is added and incubation with gentle agitation continued for 1.5-2.5 hours at 37C until a large number of protoplasts is visible in a sample inspected under the microscope.

The suspension is filtered through miracloth, the filtrate transferred to a sterile tube and overlayed with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0. Centrifugation is performed for 15 min. at 1000 g and the protoplasts are collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) are added to the protoplast suspension and the mixture is centrifugated for 5 min. at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated. Finally, the protoplasts are resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension are mixed with 5-25 µg of p3SR2 (an *A. nidulans* amdS gene carrying plasmid described in Hynes et al., Mol. and Cel. Biol., Vol. 3, No. 8, 1430-1439, Aug. 1983) in 10 µl of STC. The mixture is left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution are added and carefully mixed. The mixture is left at room temperature for 25 min., spun at 2.500 g for 15 min. and the pellet is resuspended in 2 ml of 1.2M sorbitol. After one more sedimentation the

protoplasts are spread on minimal plates (Cove, (1966), Biochem. Biophys. Acta 113, 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37C spores are picked, suspended in sterile water and spread for single colonies. This procedure is repeated and spores of a single colony after the second re-isolation are stored as a defined transformant.

Fed batch fermentation

Fed batch fermentation is performed in a medium comprising maltodextrin as a carbon source, urea as a nitrogen source and yeast extract. The fed batch fermentation is performed by inoculating a shake flask culture of *A. oryzae* host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 5.0 and 34°C the continuous supply of additional carbon and nitrogen sources are initiated. The carbon source is kept as the limiting factor and it is secured that oxygen is present in excess. The fed batch cultivation is continued for 4 days, after which the enzymes can be recovered by centrifugation, ultrafiltration, clear filtration and germ filtration. Further purification may be done by anionexchange chromatographic methods known in the art.

Purification

The culture broth is filtrated and added ammoniumsulphate (AMS) to a concentration of 1.7 M AMS and pH is adjusted to pH 5. Precipitated material is removed by centrifugation on the solution containing α-amylase activity is applied on a Toyo Pearl Butyl column previously equilibrated in 1.7 M AMS, 20 mM sodium acetate, pH 5. Unbound material is washed out with the equilibration buffer. Bound proteins are eluted with 10 mM sodium acetate, pH 4.5 using a linear gradient from 1.7 - 0 M AMS over 10 column volumes. Glucoamylase containing fractions are collected ad dialysed against 20 mM sodium acetate, pH 4.5.

Thermal Stability determination of variant of the invention

The thermal stability of variants of the invention is tested

using the following method: 950 microliter 50 mM sodium acetate buffer (pH 4.3) (NaOAc) is incubated for 5 minutes at 70°C. 50 microliter enzyme in buffer (4 AGU/ml) is added. 2 x 40 microliter samples are taken at 0 and 30 minutes and chilled on ice. The activity (AGU/ml) measured before incubation (0 minutes) is used as reference (100%). The decline in percent is calculated as a function of the incubation time.

Screening for thermostable α-amylase variants

The libraries are screened in the thermostable filter assay described below.

Filter assay for thermostability

Yeast libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on SC ura^-agar plates with 100 μg/ml ampicillin at 30°C for at least 72 hrs. The colonies are replica plated to PVDF filters (Immobilon-P, Millipore, Bedford) activated with methanol for 1 min and subsequently washed in 0.1 M NaAc and then incubated at room temperature for 2 hours. Colonies are washed from PVDF filters with tap water. Each filter sandwiches and PVDF filters are specifically marked with a needle before incubation in order to be able to localise positive variants on the filters after the screening. The PVDF filters with bound variants are transferred to a container with 0.1 M NaAc, pH 4.5 and incubated at 47°C for 15 minutes. The sandwich of cellulose acetate and nitrocellulose filters on SC ura-agar plates are stored at room temperature until use. After incubation, the residual activities are detected on plates containing 5% maltose, 1% agarose, 50 mM NaAc, pH 4.5. The assay plates with PVDF filters are marked the same way as the filter sandwiches and incubated for 2 hrs. at 50°C. After removal of the PVDF filters, the assay plates are stained with Glucose GOD perid (Boehringer Mannheim GmbH, Germany). Variants with residual activity are detected on assay plates as dark green spots on white background. The improved variants are located on the storage plates. Improved variants are rescreened twice under the

same conditions as the first screen.

General method for random mutagenesis by use of the DOPE program

The random mutagenesis may be carried out by the following steps:

1. Select regions of interest for modification in the parent enzyme,

2. Decide on mutation sites and non-mutated sites in the selected region,

3. Decide on which kind of mutations should be carried out, *e.g.* with respect to the desired stability and/or performance of the variant to be constructed,

4. Select structurally reasonable mutations,

5. Adjust the residues selected by step 3 with regard to step 4.

6. Analyze by use of a suitable dope algorithm the nucleotide distribution.

7. If necessary, adjust the wanted residues to genetic code realism, *e.g.* taking into account constraints resulting from the genetic code, *e.g.* in order to avoid introduction of stop codons; the skilled person will be aware that some codon combinations cannot be used in practice and will need to be adapted

8. Make primers

9. Perform random mutagenesis by use of the primers

10. Select resulting glucoamylase variants by screening for the desired improved properties.

Dope algorithm

Suitable dope algorithms for use in step 6 are well known in the art. One such algorithm is described by Tomandl, D. et al., 1997, Journal of Computer-Aided Molecular Design 11:29-38. Another algorithm is DOPE (Jensen, LJ, Andersen, KV, Svendsen, A, and Kretzschmar, T (1998) Nucleic Acids Research 26:697-702).

CLAIMS

1. A variant of a parent Fungamyl-like α-amylase, comprising an alteration at one or more regions selected from the group of:
Region 98-110,
Region 150-160,
Region 160-167,
Region 280-288,
Region 448-455,
Region 468-475.
wherein (a) the alteration(s) are independently
(i) an insertion of an amino acid downstream of the amino acid which occupies the position,
(ii) a deletion of the amino acid which occupies the position, or
(iii) a substitution of the amino acid which occupies the position with a different amino acid,
(b) the variant has α-amylase activity and (c) each region or position corresponds to a region position of the amino acid sequence of the parent Fungamyl-like α-amylase having the amino acid sequence of SEQ ID NO: 1.

2. The variant of claim 1, wherein the variant is one or more of the following substitution: Q152S.

3. The variant of claims 1, which variant has improved thermostability.

4. A DNA construct comprising a DNA sequence encoding a α-amylase variant of any of claims 1-3.

5. A recombinant expression vector which carries a DNA construct according to claim 4.

6. A cell which is transformed with a DNA construct according to claim 4 or a vector according to claim 5.

7. A cell according to claim 6, wherein the cell is a microorganism, such as a bacterium or a fungus.

8. The cell according to claim 7, which is a protease deficient strain of *Aspergillus,* in particular *A. oryzae*.

9. A composition for producing high maltose syrup comprising an Fungamyl-like α-amylase variant of claims 1-3.

10. The composition of claim 9 further comprising beta-amylase.

11. A dough improving composition, comprising an α-amylase variant of any of claims 1-3.

12. A brewing composition comprising an α-amylase variant of any of claims 1-3.

13. The brewing composition of claim 12, further comprising a enzymes selected from the group of beta-amylases and isoamylases.

14. A composition for producing alcohol, comprising an α-amylase variant of any of claims 1-3.

15. A process of liquefying starch, wherein an α-amylase variant of claims 1-3 is used for treating starch.

16. A process of producing high maltose syrups, wherein an α-amylase variant of claims 1-3 is used for liquefying starch.

17. A brewing process, wherein an α-amylase variant of claims 1-3 is added during fermentation of wort.

18. An alcohol production process, wherein an α-amylase variant of claim 1-3 is used for liquefaction starch in a distillery mash.

19. A process, wherein a dough product comprising an α-amylase variant of claims 1-3 is baked.

20. Use of an α-amylase variant of any of claims 1-3 or a composition of claim 9 for starch liquefaction.

21. Use of an α-amylase variant of any of claims 1-3 or a composition of claim 9 for producing alcohol.

22. Use of an α-amylase variant of any of claims 1-3 or a composition of claim 9 for brewing.

23. Use of an α-amylase variant of any of claims 1-3 or a composition of claim 9 for baking.

24. A method for generating an α-amylase variant from a parent Fungamyl-like α-amylase, which variant has improved thermostability at acidic pH relative to the parent, the method comprising:

(a) subjecting a DNA sequence encoding the parent Fungamyl-like α-amylase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a mutated α-amylase which has improved thermostability at acidic pH relative to the parent Fungamyl-like α-amylase.

Title: Fungamyl-like Alpha-Amylase Variants

ABSTRACT

The invention relates to a variant of a parent Fungamyl-like fungal α-amylase, which exhibits improved thermal stability at acidic pH suitable for, e.g., brewing processes.

Sequence Listing

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 478 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr Phe Leu Leu Thr
1               5                   10                  15

Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala Thr Cys Asn Thr
            20                  25                  30

Ala Asp Gln Lys Tyr Cys Gly Gly Thr Trp Gln Gly Ile Ile Asp Lys
        35                  40                  45

Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile Trp Ile Thr Pro
    50                  55                  60

Val Thr Ala Gln Leu Pro Gln Thr Thr Ala Tyr Gly Asp Ala Tyr His
65                  70                  75                  80

Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asn Glu Asn Tyr Gly Thr
                85                  90                  95

Ala Asp Asp Leu Lys Ala Leu Ser Ser Ala Leu His Glu Arg Gly Met
            100                 105                 110

Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly Tyr Asp Gly Ala
        115                 120                 125

Gly Ser Ser Val Asp Tyr Ser Val Phe Lys Pro Phe Ser Ser Gln Asp
    130                 135                 140

Tyr Phe His Pro Phe Cys Phe Ile Gln Asn Tyr Glu Asp Gln Thr Gln
145                 150                 155                 160

Val Glu Asp Cys Trp Leu Gly Asp Asn Thr Val Ser Leu Pro Asp Leu
                165                 170                 175

Asp Thr Thr Lys Asp Val Val Lys Asn Glu Trp Tyr Asp Trp Val Gly
            180                 185                 190

Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg Ile Asp Thr Val
        195                 200                 205

Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn Lys Ala Ala Gly
    210                 215                 220

Val Tyr Cys Ile Gly Glu Val Leu Asp Gly Asp Pro Ala Tyr Thr Cys
225                 230                 235                 240

Pro Tyr Gln Asn Val Met Asp Gly Val Leu Asn Tyr Pro Ile Tyr Tyr
                245                 250                 255

Pro Leu Leu Asn Ala Phe Lys Ser Thr Ser Gly Ser Met Asp Asp Leu
            260                 265                 270

Tyr Asn Met Ile Asn Thr Val Lys Ser Asp Cys Pro Asp Ser Thr Leu
        275                 280                 285

Leu Gly Thr Phe Val Glu Asn His Asp Asn Pro Arg Phe Ala Ser Tyr
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        290                 295                 300

Thr Asn Asp Ile Ala Leu Ala Lys Asn Val Ala Ala Phe Ile Ile Leu
305                 310                 315                 320

Asn Asp Gly Ile Pro Ile Ile Tyr Ala Gly Gln Glu Gln His Tyr Ala
                325                 330                 335

Gly Gly Asn Asp Pro Ala Asn Arg Glu Ala Thr Trp Leu Ser Gly Tyr
            340                 345                 350

Pro Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Ser Ala Asn Ala Ile
        355                 360                 365

Arg Asn Tyr Ala Ile Ser Lys Asp Thr Gly Phe Val Thr Tyr Lys Asn
    370                 375                 380

Trp Pro Ile Tyr Lys Asp Asp Ile Thr Ile Ala Met Arg Lys Gly Thr
385                 390                 395                 400

Asp Gly Ser Gln Ile Val Thr Ile Leu Ser Asn Lys Gly Ala Ser Gly
                405                 410                 415

Asp Ser Tyr Thr Leu Ser Leu Ser Gly Ala Gly Tyr Thr Ala Gly Gln
            420                 425                 430

Gln Leu Thr Glu Val Ile Gly Cys Thr Thr Val Thr Val Gly Ser Asp
        435                 440                 445

Gly Asn Val Pro Val Pro Met Ala Gly Gly Leu Pro Arg Val Leu Tyr
    450                 455                 460

Pro Thr Glu Lys Leu Ala Gly Ser Lys Ile Cys Ser Ser Ser
465                 470                 475
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